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Gid9, a second RING finger protein contributes to the ubiquitin ligase activity of the Gid complex required for catabolite degradation

Bernhard Braun [a], Thorsten Pfirrmann [b], Ruth Menssen [a], Kay Hofmann [c], Hartmut Scheel [c], Dieter H. Wolf [a,*]

[a] Institut für Biochemie, Universität Stuttgart, 70569 Stuttgart, Germany
[b] Stockholm University, Wenner-Gren Institute, S-106 91 Stockholm, Sweden
[c] Miltenyi Biotec, 51429 Bergisch-Gladbach, Germany

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ABSTRACT

The two major antagonistic pathways of carbon metabolism in cells, glycolysis and gluconeogenesis, are tightly regulated. In the eukaryotic model organism *Saccharomyces cerevisiae* the switch from gluconeogenesis to glycolysis is brought about by proteasomal degradation of the gluconeogenic enzyme fructose-1,6-bisphosphatase. The ubiquitin ligase responsible for polyubiquitylation of fructose-1,6-bisphosphatase is the Gid complex. This complex consists of seven subunits of which subunit Gid2/Rmd5 contains a RING finger domain providing E3 ligase activity. Here we identify an additional subunit containing a degenerated RING finger, Gid9/Fyv10. This subunit binds to Gid2/Rmd5. A mutation in the degenerated RING finger of Gid9/Fyv10 abolishes polyubiquitylation and degradation of three enzymes specific for gluconeogenesis.

Structured summary of protein interactions:
Gid2 physically interacts with **Gid9** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

Glucose is the most prominent carbon and energy source for many cells, being essential for mammals. Glucose is metabolized by the catabolic glycolysis pathway. When it is not available glucose must be synthesized by the anabolic pathway gluconeogenesis, which is in part a reversal of the glycolytic pathway. Irreversible and main regulatory catalytic steps of glycolysis are circumvented in gluconeogenesis by various enzymes. These steps are phosphorylation of glucose by hexokinase (glycolysis) and dephosphorylation of glucose-6-phosphate by glucose-6-phosphatase (gluconeogenesis), phosphorylation of fructose-6-phosphate by phosphofructokinase (glycolysis) and dephosphorylation of fructose-1,6-bisphosphate by fructose-1,6-bisphosphatase (FBPase) (gluconeogenesis), synthesis of pyruvate and ATP from phosphoenolpyruvate by pyruvate kinase (glycolysis) and synthesis of phosphoenolpyruvate by cytoplasmic malate dehydrogenase (c-MDH) and phosphoenolpyruvate carboxykinase (PEPCK) [1]. Malregulation of these two central antagonistic pathways in humans leads to type 2 diabetes [2]. When yeast cells are grown on a non-fermentable carbon source such as ethanol, gluconeogenesis is turned on and specific gluconeogenic enzymes such as FBPase, c-MDH and PEPCK are synthesized. Transfer of cells to glucose containing medium leads to rapid inactivation of these enzymes. The fate of FBPase has been studied best up to now. In a process called catabolite inactivation the *FBP1* gene is repressed, the enzyme is allosterically inhibited by fructose-2,6-bisphosphate and AMP and finally phosphorylated and degraded [3–10]. Two different mechanisms were published for the degradation step [11]. After glucose addition of cells grown and starved for 48 h on acetate as non-fermentable carbon source a vacuolar degradation of FBPase was reported. This process is dependent on the uptake of FBPase into vesicles and requires so-called Vid proteins [12–14]. In contrast, glucose addition to cells grown on ethanol for 16–18 h results in rapid degradation of the enzyme by the ubiquitin proteasome pathway (UPS) called catabolite degradation [7,11,15–20]. Likewise, degradation of PEPCK was shown to follow the proteasomal degradation route [20]. In addition, proteasomal degradation of FBPase and PEPCK requires the Hsp70 chaperone Ssa1 as well as the Cdc48-Ufd1-Npl4 motor complex [21–23]. Ubiquitin triggered degradation of a protein is initiated by a polyubiquitin chain linked to a lysine residue or the amino terminus of the respective protein [24]. This requires activation of the 76 amino acid ubiquitin by an ubiquitin activating enzyme

* Corresponding author. Address: Universität Stuttgart, Institut für Biochemie, Pfaffenwaldring 55, 70569 Stuttgart, Germany . Fax: +49 711 68564392.
E-mail address: dieter.wolf@ibc.uni-stuttgart.de (D.H. Wolf).

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Table 1
Strains used in this study.

Strain	Genotype	Source
YWO0903	W303-1B MATalpha, *ade2 leu2-3, 112 his3 trp1 ura3*	H.L. Chiang
YWO0986	W303 MATalpha, *ade2 leu2-3, 112 his3 trp1 ura3 GID9-HA$_3$::HIS5$^{S.pombe}$*	T. Pfirrmann
Y31488	MATa/alpha, *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0 gid9Δ::KANMX4/gid9Δ::KANMX4*	EUROSCARF
YRM49b	W303-1B *gid1Δ::loxP gid2Δ::loxP gid4Δ::loxP gid5Δ::loxP gid7Δ::loxP gid8Δ::loxP gid9Δ::loxP*	R. Menssen
YWO1850	W303-1B *gid1Δ::loxP gid2Δ::GID2-V5-HIS3$^{S.pombe}$ gid4Δ::loxP gid5Δ::loxP gid7Δ::loxP gid8Δ::loxP gid9Δ::loxP*	J. Schweiggert
YWO1978	W303-1B *gid1Δ::loxP gid2Δ::GID2ΔCTLH-V5-HIS3$^{S.pombe}$ gid4Δ::loxP gid5Δ::loxP gid7Δ::loxP gid8Δ::loxP gid9Δ::loxP*	This study
YWO1979	W303-1B *gid1Δ::loxP gid2Δ::GID2ΔLisH-V5-HIS3$^{S.pombe}$ gid4Δ::loxP gid5Δ::loxP gid7Δ::loxP gid8Δ::loxP gid9Δ::loxP*	This study
YWO1255	W303-1B *gid9Δ::KANMX4*	M. Lehmann

Table 2
Plasmids used in this study.

Plasmid	Characteristics	Backbone	Source
PWO0747	3.2kbp DNA fragment containing Gid9 with its native promotor and terminator	pRS316	T. Pfirrmann, B. Braun
PWO1315	3.2kbp DNA fragment containing Gid9C434S with its native promotor and terminator	pRS316	This study
pJD421	His$_6$-Ubiquitin inserted between P$_{CUP1}$ and T$_{CYC1}$	YEplac181	J. Dohmen
PWO1283	Gid2-V5, genomic promotor, ADH1 terminator	pRS303	J. Schweiggert
PWO1313	*gid2Δ216-229CTLH-V5*	pRS303	This study
PWO1314	*gid2Δ138-171LisH-V5*	pRS303	This study
PWO1153	Gid2-HA$_3$, native promotor, ADH1 terminator	pRS316	L. Barbin
PWO1316	*gid2Δ216-229CTLH-HA$_3$*	pRS316	This study with help of E. Diler
PWO1317	*gid2Δ138-171LisH-HA$_3$*	pRS316	This study

Table 3
Primers used in this study.

Primers	
Gid9fwd	aaagaattcgaaagcgagaagtataca
Gid9rev	aaaaactcgagaacttaatcaagggaagg
Gid9*RING fwd	cgctacattcactaaaaaggaagaattctcccgtttgcagtgagac
Swa1-Pme1	gctataaaaataattatagtttaaacttttaatataaatatata
ED 3	ctaaccatacggctttatcgtccaagcatggaaatccggtagag
ED 4	ctctaccggatttcgcttggacgataaagccgtatggttag
ED 9	Aaaatcgatttcgccccagtaccacggttccttaccaa
ED 10	gtcagtcgacctgttatccctagcggatct
Gid2*LisHfwd	gagacaatggaaaatgttaatgaaaaagaatctactgaatttattgagatg
Gid2*LisHrev	catctcaataaattcagtagattcaaattcattaacattttccattgtctc
RM83	tatagtcgacccgctatcagagatggaccg
RM84	tatagcggccgcactacaaaaagcaccaaggcc

(E1) resulting in a thioester bond between the C-terminal glycine of ubiquitin and a cysteine residue of the E1. Transfer of ubiquitin to an ubiquitin conjugating enzyme (E2) follows. Subsequently ubiquitin is transferred to the target protein in a concerted action of the E2 and ubiquitin ligases (E3's). While HECT domain E3 ligases first receive the ubiquitin from the E2 and then transfer it to the protein to be degraded, RING domain E3 ligases bind the E2 as well as the substrate and catalyse the direct transfer of the ubiquitin from the E2 to the substrate [25]. The responsible ubiquitin ligase for FBPase degradation, the Gid (glucose induced degradation deficient) complex, was discovered [18]. This E3 ligase complex is composed of 7 subunits of which Gid2 contains a RING finger domain, which confers ubiquitin ligase activity to the complex *in vitro* and *in vivo* [20]. In addition, the two other

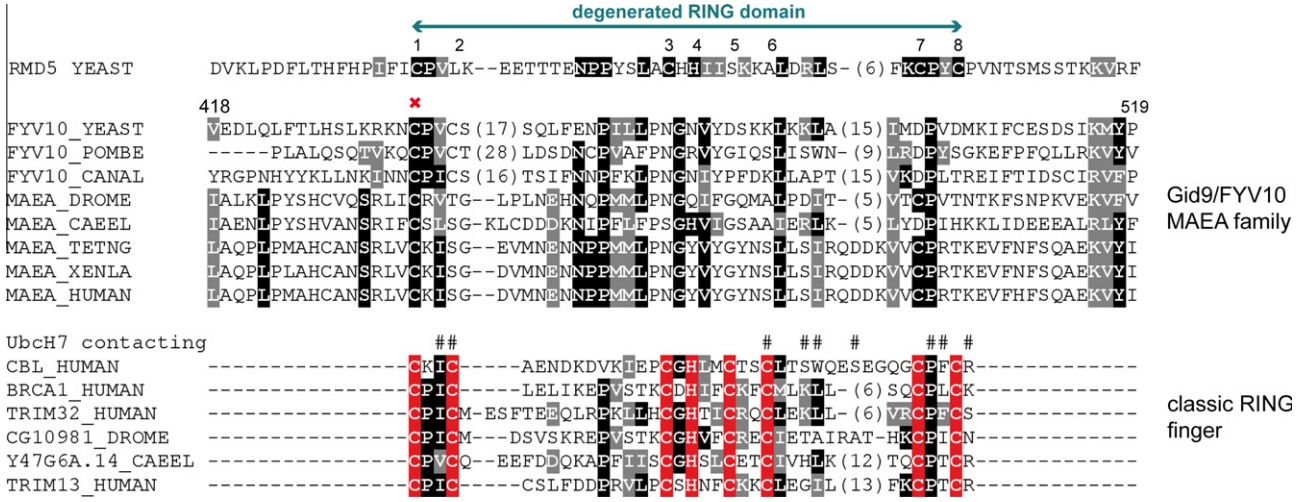

Fig. 1. RING domain alignment of the Gid9 protein family with Gid2 and classic RING-finger proteins. Black or grey background labels amino acid positions with invariant or conservatively replaced residues in at least 50% of the sequences. Numbers (1–8) of the alignment show positions homologous to zinc binding residues in classic RING finger proteins. A red cross marks the position of a point mutation introduced into the degenerated RING domain of Gid9 (C434S). # marks the residues essential for interaction between Cbl and UbcH7 [44]. MAEA: macrophage erythroblast attacher. Species abbreviation: YEAST, *S. cerevisiae*; POMBE, *S. pombe*; CANAL, *Candida albicans*; DROME, *Drospophila melanogaster*; CAEEL, *Caenorhabditis elegans*; TETNG, *Tetraodon nigroviridis*; XENLA, *Xenopus laevis*; HUMAN, *H. sapiens*.

gluconeogenic enzymes PEPCK and c-MDH are also targets of the E3 complex [20, this paper]. So far, no other proteins than the gluconeogenic enzymes have been found to be ubiquitylated by the Gid complex. A similar complex, the so called CTLH complex, of mainly unknown function was found in mammalian cells [20,26]. Interestingly, some of the Gid proteins were also identified as Vid proteins required for vesicle and vacuole dependent degradation of FBPase under harsh starvation conditions on acetate. However, no specific biochemical function has been assigned to these proteins in this process [27,28]. Here we show that besides Gid2 a second subunit of the Gid complex, Gid9, contains a degenerated RING finger domain. When this domain is mutated, the three gluconeogenic enzymes FBPase, PEPCK and c-MDH fail to be ubiquitylated and degraded. Gid9 binds to Gid2 in the absence of other Gid proteins. The two subunits seem to form the heterodimeric E3 ligase unit of the Gid complex.

2. Materials and methods

2.1. Yeast strains, plasmids and media

Previously described standard methods were used for media preparation and genetic and molecular biological techniques [29]. Yeast strains were grown at 30 °C. Precultures were grown 16 h in YPD or CM medium containing 2% glucose, diluted 1:12.5 into YPD or CM and grown for additional 6–7 h. Thereafter, cells were resuspended in YPethanol or CM (2% ethanol) and grown for 16 h to allow FBPase synthesis (final OD_{600} 1-2). For induction of FBPase degradation cells were shifted to YPD or CM medium containing 2% glucose.

For construction of strain YTP12 see [20]. Strain Y31488 (*Δgid9*) was obtained from the EUROSCARF collection.

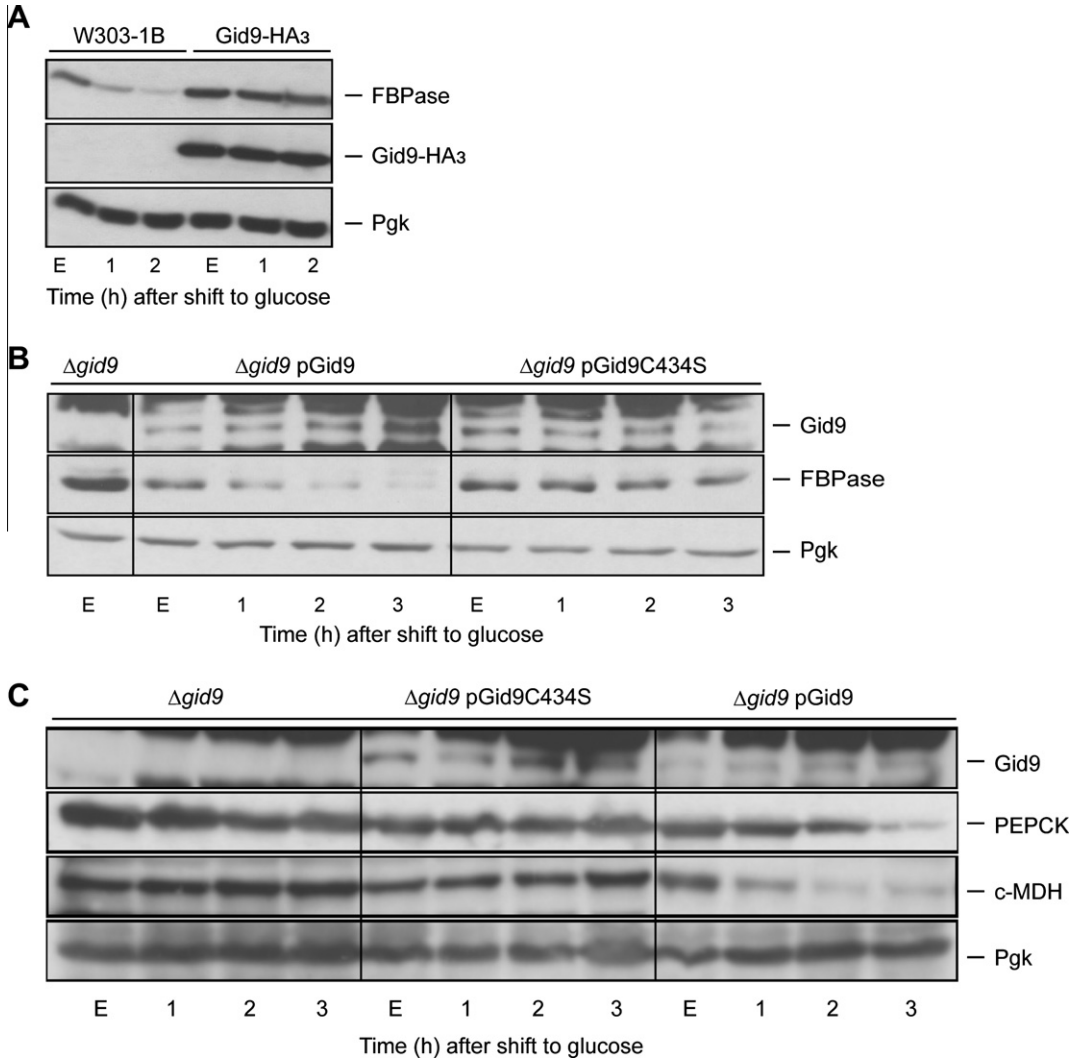

Fig. 2. (A) A C-terminal HA_3 tag renders Gid9 inactive. Cells were grown 16 h at 30 °C in YPethanol and then shifted into YPD medium. Cell samples were taken at the indicated time points, lysed and after SDS PAGE proteins were visualized via immunoblotting. Loading control: Pgk, 3-phosphoglycerate kinase. (B) A mutation in the putative RING domain of Gid9 (Gid9C434S) leads to inactivation of the protein with respect to FBPase degradation. Gid9 and its mutated C434S counterpart were expressed in a *Δgid9* strain from plasmid pRS316. Cells were grown 16 h at 30 °C in CM medium with ethanol as carbon source without uracil and shifted to the same medium containing glucose instead of ethanol. At the indicated time points 1.5 OD of cells were harvested and after cell lysis and SDS PAGE proteins were monitored by immunoblotting. Loading control: Pgk, 3-phosphoglycerate kinase. (C) A mutation in the putative RING domain of Gid9 (Gid9C434S) leads to inactivation of the protein with respect to degradation of PEPCK and c-MDH. Gid9 and its mutated C434S counterpart were expressed in a *Δgid9* strain from plasmid pRS316. Cells were grown 16 h at 30 °C in CM medium with ethanol as carbon source without uracil and shifted to the same medium containing glucose instead of ethanol. At the indicated time points 1.5 OD of cells were harvested, lysed and after SDS PAGE and proteins were monitored by immunoblotting. Loading control: Pgk, 3-phosphoglycerate kinase.

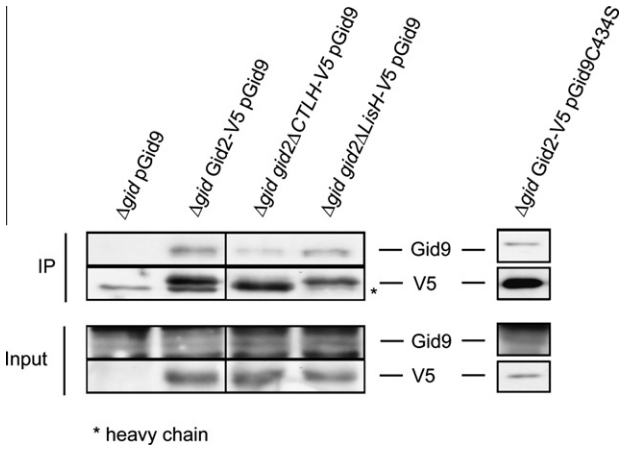

Fig. 3. Gid2 and Gid9 interact. A strain, *Δgid*, deleted in all Gid subunits (*GID1*, *GID2*, *GID4*, *GID5*, *GID7*, *GID8* and *GID9*) and chromosomally expressing *GID2-V5*, *gid2Δ216-229CTLH-V5* or *gid2Δ138-171LisH-V5* respectively, was transformed with plasmid pWO 0747 expressing *GID9*. Cells were grown 16 h on CM medium with ethanol as carbon source and without uracil. Samples were taken and immunoprecipitation was performed with V5 antibody. Proteins were visualized by immunodetection with V5 and Gid9 antibodies.

Strain YRM49b (*Δgid*, deletion of *GID1,2,4,5,7,8,9*) was generated using the technique described (R. Menssen, in preparation); [30]. *Δgid GID2-V5*, *Δgid gid2Δ216-229CTLH-V5* and *Δgid gid2Δ138-171LisH-V5* strains were generated by integration of Sfo1 digested plasmids pWO1283, pWO1313 and pWO1314 into YRM49b.

The plasmids expressing wildtype Gid9 or RING finger mutated Gid9C434S were constructed by insertion of a *GID9* PCR-fragment (primers Gid9fwd, Gid9rev) with its endogenous promotor and terminator regions in a Xho1/EcoR1 digested pRS316 plasmid [31]. Point mutation of the preserved Cys residue 434 of Gid9 was performed using the Transformer site-directed mutagenesis kit (Clontech, Mountain View, CA) (primers Gid9*RING fwd, Swa1-Pme1). The plasmid JD421 expressing poly-histidine-tagged Ub was obtained from Dohmen et al. [32]. PWO1153 [33] was used as template plasmid for construction of CTLH domain deleted pWO1316 (primers ED3, ED4, ED9, ED10) and LisH domain deleted pWO1317 (primers Gid2*LisHfwd, Gid2*LisHrev, ED9, ED10). A cloned PCR fragment from strain YWO1850 obtained with primers RM83 and RM84 was inserted into Sal1/Not1 digested pRS303 resulting in plasmid pWO1283. Tables 1–3 list the yeast strains, plasmids and primers used.

2.2. FBPase/c-MDH/PEPCK ubiquitylation analysis

Ubiquitylation assays were performed by growing cells with a plasmid encoding poly-histidine-tagged ubiquitin (pJD421). Expression of His_6-Ub was induced by adding $CuSO_4$ to a final concentration 0.2 mM. Cells were grown 16-22 h in CM medium with ethanol as carbon source without uracil and leucine to allow derepression of FBPase. Thereafter, 150 OD_{600} of yeast cells were harvested before and 30 min after addition of 2% glucose. After washing in 1 ml of water, cells were frozen in liquid N_2 and stored at -80 °C. Cells were then further processed as described in [34].

2.3. Western blotting

Western blotting was carried out as described in ref. [17] and [20]. Antibodies used were obtained from BAbCO (Richmond, CA) (hemagglutinin [HA], clone 16B12); Sigma Aldrich (Schnelldorf), (V5). FBPase, PEPCK and c-MDH polyclonal antibodies were pro-

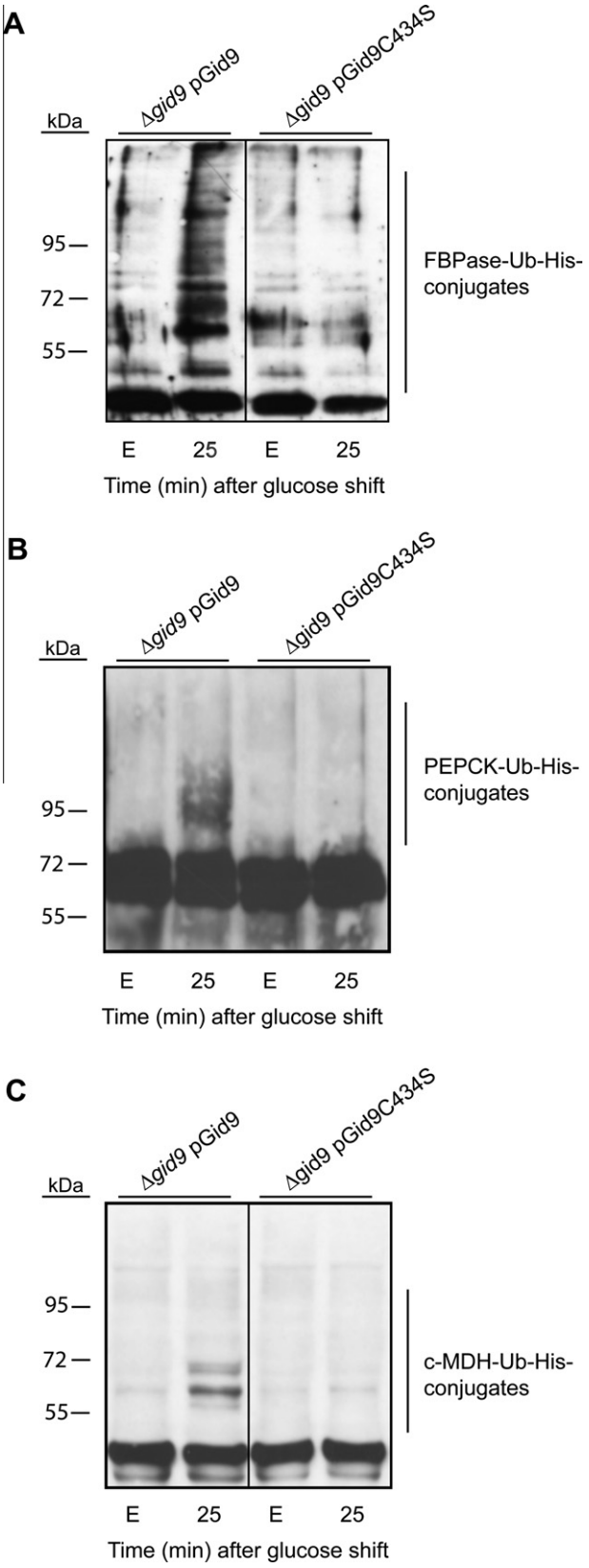

Fig. 4. Polyubiquitylation of gluconeogenic enzymes is abolished in strains expressing Gid9C434S. A *Δgid9* strain expressing plasmid encoded wild-type Gid9 or mutant Gid9C434S as well as poly-histidine-tagged ubiquitin were grown for 16–22 h in CM medium without uracil and leucine, 2% ethanol as carbon source and shifted onto the same medium with 2% glucose instead of ethanol. Samples were taken at indicated time points and poly-histidine tagged ubiquitin was precipitated with Ni-NTA-Sepharose beads. Polyubiquitylation was detected using specific antibody against (A) FPBase, (B) PEPCK and (C) c-MDH.

duced by immunization of rabbits using a purified FBPase-/PEPCK-/c-MDH-glutathione transferase (GST). Gid9 antibody was produced by immunization of rabbits using a Gid9 peptide (amino acid 471 to 486 of the Gid9 sequence; Charles River).

2.4. Co-immunoprecipitation

For immunoprecipitations (IPs) cells were cultivated as described above and samples were taken at the indicated time points. Cells (50-70 OD_{600}) were harvested, washed with water and resuspended in 600 µl of phosphate-buffered saline (PBS) buffer pH 7.4 (137 mM NaCl, 1.25 g/l Na_2HPO_4, and 0.35 g/l NaH_2PO_4) containing protease inhibitors (ProteoBlock PIC, Thermo Scientific, Fermentas Molecular Biology Tools; 1.1 mM phenylmethyl-sulfonyl-fluoride (PMSF), 0.1-0.2% Triton-X-100) and lysed at 8 °C with glass beads for 15 min. After centrifugation, 500 µl of the supernatant were transferred to a new test tube. 0.8 µl V5-antibody was added and the samples were gently agitated end over end for 2 h at 8 °C. Immunoprecipitates were collected by adding 50 µl of 6% (wt/vol) protein A-Sepharose CL-4B (GE Healthcare, Little Chalfont, United Kingdom) and further incubated for 1.5 h. For IP the Sepharose beads were centrifuged and washed five times with ice-cold PBS buffer. Proteins were released from Sepharose by boiling in 50 µl of urea buffer.

Bioinformatic sequence analysis was performed as indicated in [20].

3. Results and discussion

Recently, Gid2 was identified as a RING finger protein providing ubiquitin ligase activity to the seven subunit containing Gid complex. In several cases it has been shown that RING finger E3 ligases exert their optimum activity in a dimeric state whereby homodimers and heterodimers have been found [25,35–42]. We therefore searched whether the Gid-ubiquitin ligase complex harbors a second subunit which besides Gid2 contains a RING-finger motif. When aligning the amino acid sequences of yeast Gid proteins with the sequence of yeast Gid2, the sequences of the MAEA protein family, formerly found to be mammalian orthologues of Gid9 [20], as well as classical RING finger proteins, Gid9 emerges carrying a degenerated RING finger domain with two conserved residues (Fig. 1). For analysis of the function of Gid9 in the Gid complex we fused a triple HA tag onto the C-terminus of Gid9. However, the Gid9-HA_3 fusion proved to be not functional: the protein failed to trigger degradation of FBPase when cells were transferred from gluconeogenic to glycolytic growth conditions. Obviously HA-tagging of Gid9 results in a biochemically inactive Gid9 mutant (Fig. 2A). The outcome of the experiment indicated a central function for a structurally intact Gid9 protein in the Gid-complex. We therefore raised antibodies against Gid9 in rabbits to follow its expression and to monitor the interaction of Gid9 with Gid2, which had been shown to exhibit ubiquitin ligase activity [20]. For this monitoring purpose we tagged Gid2 with the V5 tag. The fusion protein is active *in vivo* (not shown). For monitoring an interaction of Gid9 with Gid2-V5 we expressed both proteins in a strain deleted in all seven Gid proteins, which is fully vaible but fails to degrade the gluconeogenic enzymes. As can be seen in Fig. 3, when precipitating Gid2 with V5 antibodies, Gid9 co-precipitates. Clearly, Gid2 and Gid9 interact in the Gid complex. In addition to its RING domain Gid2 contains a CTLH and a LisH domain [20]. Deletion of these domains slows down FBPase degradation (not shown). We asked whether any of these domains were involved in the binding to Gid9. When expressing Gid2-V5 deleted in its CTLH domain or in its LisH domain together with Gid9 in the strain devoid of all other Gid proteins, both Gid2 domain mutant proteins co-precipitated with Gid9 upon application of V5 antibodies. This indicates that these domains are not essential for the Gid2-Gid9 interaction even though the interaction was somehow less pronounced when the CTLH domain in Gid2 was deleted (Fig. 3). The fact that tagging of Gid9 results in stabilization of FBPase indicates that Gid9 has a crucial function in the degradation process (Fig. 2A). We therefore introduced a mutation into the

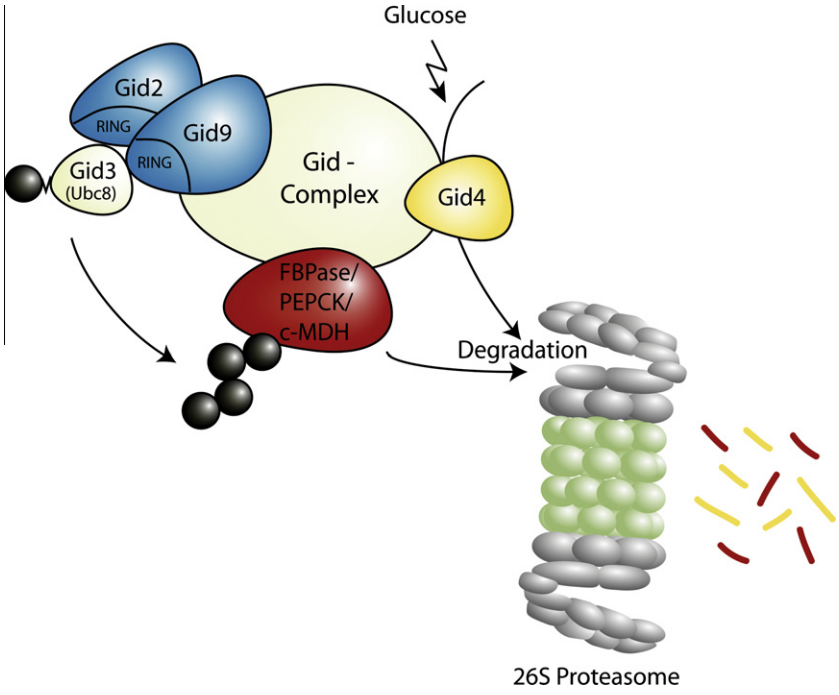

Fig. 5. The Gid complex triggers polyubiquitylation and subsequent proteasomal degradation of the gluconeogenic enzymes FBPase, PEPCK and c-MDH. After addition of glucose, Gid4 appears and activates the Gid E3 ligase. The Gid2 and Gid9 dimer together with Gid3, the ubiquitin conjugating enzyme Ubc8, polyubiquitylate the substrate proteins and by this trigger their degradation by the proteasome. (modified from [20]).

degenerated RING finger domain of Gid9 by changing the conserved cysteine residue 434 to serine to inactivate the RING domain[20,43]. When testing the consequence of the Gid9C434S mutation on the catabolite degradation of FBPase, it was obvious that degradation of the enzyme is blocked (Fig. 2B). Also catabolite degradation of the gluconeogenic enzymes PEPCK and c-MDH is abrogated by the mutation (Fig. 2C). As the Gid-complex acts as the ubiquitin ligase in the proteasome catalyzed degradation process of these three enzymes, we expected that ubiquitylation is disturbed by the C434S mutation in Gid9. This is indeed the case: expression of the mutated Gid9C434S protein in a strain otherwise deleted for *GID9* leads to a lack in polyubiquitylation of FBPase (Fig. 4A), PEPCK (Fig. 4B) and c-MDH (Fig. 4C), when cells are shifted from gluconeogenic to glycolytic conditions, by this preventing subsequent proteasomal degradation (Fig. 2B and C). We were not able to show any Gid9 ligase activity *in vitro* (not shown). Interestingly the C434S mutation in Gid9 does not disturb binding of the protein to Gid2 (Fig. 3). This excludes a defective complex formation between Gid2 and Gid9 as the cause of failure of the Gid-complex harboring Gid9C434S to polyubiquitylate and degrade FBPase, PEPCK and c-MDH. Clearly, an intact Gid9 protein has an important impact on the polyubiquitylating activity of the Gid-ubiquitin ligase complex. A model proposing the sequence of events of the catabolite degradation process is depicted in Fig. 5.

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